

	Type	Hits	Search Text	DBs
1	BRS	426	myceliophthora near2 thermophila	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
2	BRS	334	S101 and laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
3	BRS	325	S102 and (mutant or variant or modified or modification or mutation or substitution)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
4	BRS	314	S103 and ("93" or "106" or "108" or "109" or "48" or "433" or "500" or "506" or "510" or "514")	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
5	BRS	160	S103 and ("93" or "106" or "108" or "109" or "48" or "433" or "500" or "506" or "510" or "514")	USPAT; EPO
6	BRS	249	S101 near10 laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
7	BRS	121	S106 and ("93" or "106" or "108" or "109" or "48" or "433" or "500" or "506" or "510" or "514")	USPAT; EPO
8	BRS	127	S101 near10 laccase	USPAT; EPO
9	BRS	433	myceliophthora near2 thermophila	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
10	BRS	340	S109 and laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
11	BRS	330	S110 and (mutant or variant or modified or modification or mutation or substitution)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
12	IS&R	3	((("6184015") or ("5985818"))).PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
13	IS&R	16	((("6060442") or ("5998353") or ("6184015") or ("6218170") or ("6277611") or ("5972670") or ("5795760") or ("5948121") or ("5981243"))).PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
14	BRS	5	"5998353"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB

	Time Stamp
1	2007/07/31 10:07
2	2006/04/25 13:24
3	2006/06/14 12:22
4	2006/04/25 13:24
5	2006/04/25 13:24
6	2006/04/25 13:34
7	2006/04/25 13:24
8	2006/04/25 13:34
9	2006/06/14 12:22
10	2006/06/14 12:22
11	2006/06/14 12:43
12	2006/06/14 12:36
13	2006/06/14 12:49
14	2006/06/14 12:46

	Type	Hits	Search Text	DBs
15	BRS	3	"5925554"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
16	BRS	48	laccase near10 detergent	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
17	BRS	222	S118 and (variant or mutant or modified or mutation or modification)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
18	BRS	242	laccase near20 detergent	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
19	BRS	932	laccase and detergent	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
20	BRS	2768	laccase	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
21	BRS	5	"5998353"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
22	BRS	386	S123 and (mutant or variant or modified or modification or mutation)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
23	BRS	446	S120 and thermophila	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
24	BRS	402	S122 and myceliophthora	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
25	BRS	360	S124 and (myceliophthora near2 thermophila)	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
26	BRS	8	"5985818"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
27	IS&R	20	((("5925554") or ("5985818") or ("5998353") or ("6060442") or ("6559859") or ("6184015") or ("6218170") or ("6277611") or ("5770419") or ("5972670") or ("5795760"))).PN.	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
28	BRS	4	"7,056,714"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB
29	BRS	3	"7,109,010"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM_TDB

	Time Stamp
15	2006/06/14 12:46
16	2006/06/14 13:58
17	2006/06/14 13:58
18	2006/06/14 13:58
19	2006/06/14 13:57
20	2006/12/11 14:30
21	2006/12/11 14:38
22	2006/12/11 14:40
23	2006/12/11 14:39
24	2006/12/11 14:39
25	2006/12/11 14:41
26	2006/12/11 14:45
27	2006/12/18 08:46
28	2006/12/11 15:27
29	2006/12/11 15:27

	Type	Hits	Search Text	DBs
30	BRS	3	"20030074698"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM TDB
31	BRS	3	"20050044594"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM TDB
32	BRS	3	"6140092"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM TDB
33	BRS	10	"6218170" or "6060442"	US-PGPUB; USPAT; EPO; JPO; DERWENT; IBM TDB
34	IS&R	5	((("5925554") or ("5998353") or ("6559859") or ("6184015") or ("5972670"))).PN.	USPAT; USOCR
35	IS&R	1	("6140092").PN.	USPAT; USOCR
36	IS&R	5	((("5925554") or ("5998353") or ("6559859") or ("6184015") or ("5972670"))).PN.	USPAT; USOCR
37	BRS	2	11 and surfactant	USPAT
38	BRS	3	11 and detergent	USPAT
39	BRS	2	14 and (cleaning or surfactant or detergent)	USPAT
40	BRS	2	"6140092"	USPAT

	Time Stamp
30	2006/12/12 14:27
31	2006/12/12 14:27
32	2006/12/18 08:46
33	2007/07/24 14:16
34	2007/07/31 12:48
35	2007/07/31 10:10
36	2007/07/31 12:48
37	2007/07/31 12:49
38	2007/07/31 12:58
39	2007/07/31 12:59
40	2007/07/31 13:08

RESULT 3

LAC2_PODAN

ID LAC2_PODAN STANDARD; PRT; 621 AA.

AC P78722;

DT 01-NOV-1997, integrated into UniProtKB/Swiss-Prot.

DT 01-MAY-1997, sequence version 1.

DT 07-FEB-2006, entry version 42.

DE Laccase II precursor (EC 1.10.3.2) (Benzenediol:oxygen oxidoreductase)

DE (Urishiol oxidase) (Diphenol oxidase) (Laccase C).

GN Name=LAC2;

OS Podospora anserina.

OC Eukaryota; Fungi; Ascomycota; Pezizomycotina; Sordariomycetes;

OC Sordariomycetidae; Sordariales; Lasiosphaeriaceae; Podospora.

OX NCBI_TaxID=5145;

RN [1]

RP NUCLEOTIDE SEQUENCE [GENOMIC DNA].

RC STRAIN=ATCC 26003;

RX MEDLINE=97071669; PubMed=8914515; DOI=10.1007/s004380050261;

RA Fernandez-Larrea J., Stahl U.;

RT "Isolation and characterization of a laccase gene from Podospora

RT anserina.";

RL Mol. Gen. Genet. 252:539-551(1996).

CC -!- FUNCTION: Probably involved in lignin degradation and in the

CC detoxification of lignin-derived products in its natural habitat

CC (herbivorous dung), which is rich in lignin of grasses and straw.

CC Probably involved in melanin synthesis and in perithecia

CC development.

CC -!- CATALYTIC ACTIVITY: 4 benzenediol + O(2) = 4 benzosemiquinone + 2

CC H(2)O.

CC -!- COFACTOR: Binds 4 copper ions per monomer (By similarity).

CC -!- SUBUNIT: Monomer.

CC -!- SUBCELLULAR LOCATION: Secreted protein.

CC -!- DEVELOPMENTAL STAGE: Low basic levels throughout the growth phase;

CC increases at least 20-fold at the beginning of the autolytic phase

CC and decreases again thereafter.

CC -!- INDUCTION: Under oxidative stress on the mycelium by aromatic

CC xenobiotics (guaiacol, hydroquinone, benzoquinone), and by copper

CC salt at a concentration of 1mM (growing mycelium).

CC -!- PTM: Proteolytically processed at both its N-terminus and its C-

CC terminus.

CC -!- MISCELLANEOUS: Podospora anserina contains at least 3 laccase

CC isozymes named I, II, and III. They differ in their substrate

CC specificity, number of subunits, isoelectronic point and heat

CC stability.

CC -!- SIMILARITY: Belongs to the multicopper oxidase family.

```

CC  -!- SIMILARITY: Contains 3 plastocyanin-like domains.
CC  -----
CC  Copyrighted by the UniProt Consortium, see http://www.uniprot.org/terms
CC  Distributed under the Creative Commons Attribution-NoDerivs License
CC  -----
DR  EMBL; Y08827; CAA70061.1; -; Genomic_DNA.
DR  PIR; S72493; S72493.
DR  HSSP; Q9Y780; 1HFU.
DR  SMR; P78722; 46-605.
DR  InterPro; IPR001117; Cu-oxidase.
DR  InterPro; IPR011706; Cu-oxidase_2.
DR  InterPro; IPR011707; Cu-oxidase_3.
DR  InterPro; IPR002355; Cu_oxidase_Cu_BS.
DR  Pfam; PF00394; Cu-oxidase; 1.
DR  Pfam; PF07731; Cu-oxidase_2; 1.
DR  Pfam; PF07732; Cu-oxidase_3; 1.
DR  PROSITE; PS00079; MULTICOPPER_OXIDASE1; 1.
DR  PROSITE; PS00080; MULTICOPPER_OXIDASE2; 1.
KW  Copper; Glycoprotein; Lignin degradation; Melanin biosynthesis;
KW  Metal-binding; Oxidoreductase; Repeat; Signal.
FT  SIGNAL          1      23      Potential.
FT  PROPEP          24      48      Potential.
FT                                     /FTId=PRO_0000002931.
FT  CHAIN           49     605      Laccase II.
FT                                     /FTId=PRO_0000002932.
FT  PROPEP          606     621      Potential.
FT                                     /FTId=PRO_0000002933.
FT  DOMAIN           78     201      Plastocyanin-like 1.
FT  DOMAIN          210     367      Plastocyanin-like 2.
FT  DOMAIN          430     566      Plastocyanin-like 3.
FT  METAL           138     138      Copper 1 (type 2) (By similarity).
FT  METAL           140     140      Copper 2 (type 3) (By similarity).
FT  METAL           183     183      Copper 2 (type 3) (By similarity).
FT  METAL           185     185      Copper 3 (type 3) (By similarity).
FT  METAL           476     476      Copper 4 (type 1) (By similarity).
FT  METAL           479     479      Copper 1 (type 2) (By similarity).
FT  METAL           481     481      Copper 3 (type 3) (By similarity).
FT  METAL           548     548      Copper 3 (type 3) (By similarity).
FT  METAL           549     549      Copper 4 (type 1) (By similarity).
FT  METAL           550     550      Copper 2 (type 3) (By similarity).
FT  METAL           554     554      Copper 4 (type 1) (By similarity).
FT  CARBOHYD        133     133      N-linked (GlcNAc. . .) (Potential).
FT  CARBOHYD        261     261      N-linked (GlcNAc. . .) (Potential).
FT  CARBOHYD        276     276      N-linked (GlcNAc. . .) (Potential).
FT  CARBOHYD        289     289      N-linked (GlcNAc. . .) (Potential).

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FT	CARBOHYD	325	325	N-linked (GlcNAc. . .) (Potential).
FT	CARBOHYD	334	334	N-linked (GlcNAc. . .) (Potential).
FT	CARBOHYD	401	401	N-linked (GlcNAc. . .) (Potential).
FT	CARBOHYD	421	421	N-linked (GlcNAc. . .) (Potential).
FT	CARBOHYD	441	441	N-linked (GlcNAc. . .) (Potential).
SQ	SEQUENCE	621 AA;	68129 MW;	79F85E2ED25C1CA7 CRC64;

Query Match 70.7%; Score 2231.5; DB 1; Length 621;
 Best Local Similarity 68.9%; Pred. No. 5.6e-150;
 Matches 397; Conservative 65; Mismatches 109; Indels 5; Gaps 4;

Qy	1	QQSCNTPSNRACWTDGYDINTDYEVDSPDTGVVRPYTLTLTEVDNWTGPDGVVKEKVMLV	60
		: : : : :	
Db	46	QSSCHTAANRACWAPGFDINTDYEVSPTNTGVTRTYTLTLTEVDNWLGPDGVVKQKVMLV	105
Qy	61	NNSIIGPTIFADWGDITQVTVINNLETNGTSHHWHGLHQKGTNLHDGANGITECPIPPKG	120
		: : :	
Db	106	NGDIFGPTITANWGDWIQVNVINNLRNTNGTSHHWHGLHQKGTNMHDGANGVTECPIPPKG	165
Qy	121	GRKVYRFKAQQYGTSWYHSHFSAQYGNGVVGAIQINGPASLPYDIDLGVFPISDYYYSSA	180
		: : : : :	
Db	166	GSRIYRFRAQQYGTSWYHSHFSAQYGNGVVGTVVNGPASVPYDIDLGVFPITDYYHKPA	225
Qy	181	DELVELTKNSGAPFSDNVLFNGTAKHPETGEGEYANVTLTGRRHRLRLINTSVENHFQV	240
		: : : : : :	
Db	226	DVLVEETMNGGPPPSDTVLFKGHGKNPQTGAGKFANVTLTGKRHRLRIINTSTHDFHQL	285
Qy	241	SLVNHTMCIIAADMVPVNMAMTVDSLFLGVGQRYDVVIEANRTPGNYWFNVTFGGGLLCGG	300
		: :	
Db	286	KLQNHTMTIIAADMVPVQAQTVDSLFLAVGQRYDVTIDANKSVGNYWFNATFGGGLACGA	345
Qy	301	SRNPYPAAIFHYAGAPGGPPTDEGKAPVDHNCCLDLPNLKPVVARDVPLSGFAKRADNTLD	360
		: : : : :	
Db	346	SLNPHPAAVFRYQGAPNTLPTNIGTPAADANCMDLNNLTPVVSRSVPTSGFTPRPNNTLP	405
Qy	361	VTLDTTGTPLFVWKVNGSAINIDWGRAVDYVLTQNTSFPPGYNIVEVNGADQWSYWLIE	420
		: : : : : : :	
Db	406	VSLTLGGTPLFVWKVNGSSINVDWDKPIVDYVIAQNTSYPPQANVITVNSVNQWTYWLIE	465
Qy	421	NDPGAPFTLPHPMHLHGHDYVLGRSPDESPAS--NERHVFDPARDAGLLSGANPVRRDV	478
		: : : : : :	
Db	466	NDPTGPFSIPHPMHLHGHDYVLVGRSPDQ-PAGVPQTRYRFNPATDMALLKSSNPVRRDV	524
Qy	479	SMLPAFGWVVLSEFRADNPGAWLFHCHIAWHVSGGLGVVYLERADDLRGAVSDADADDLDR	538
		: : : : : : : :	

Db 525 AMLPANGWLLIAFKSDNPGAWLFHCHIAWHVSGGLSVQYLERPNDLRNGFSQADKNQHNN 584

Qy 539 LCADWRRYWPTNPYPKSDSGLK-HRWV-EEGEWLVK 572

Db 585 NCNAWRAYWPTNPFPKIDSGLKVKKWVGEHPDWYIK 620

RESULT 2

KSNCLO
laccase (EC 1.10.3.2) precursor - *Neurospora crassa* (strain OR)
N;Alternate names: urishiol oxidase
C;Species: *Neurospora crassa*
C;Date: 30-Sep-1991 #sequence_revision 30-Sep-1991 #text_change 09-Jul-2004
C;Accession: A28523; A29762
R;Germann, U.A.; Mueller, G.; Hunziker, P.E.; Lerch, K.
J. Biol. Chem. 263, 885-896, 1988
A;Title: Characterization of two allelic forms of *Neurospora crassa* laccase.
Amino- and carboxyl-terminal processing of a precursor.
A;Reference number: A28523; MUID:88087214; PMID:2961749
A;Accession: A28523
A;Molecule type: DNA
A;Residues: 1-619 <GER>
A;Cross-references: UNIPROT:P06811; EMBL:M14554
R;Germann, U.A.; Lerch, K.
Proc. Natl. Acad. Sci. U.S.A. 83, 8854-8858, 1986
A;Title: Isolation and partial nucleotide sequence of the laccase gene from
Neurospora crassa: amino acid sequence homology of the protein to human
ceruloplasmin.
A;Reference number: A29762; MUID:87067412; PMID:2947240
A;Accession: A29762
A;Molecule type: DNA
A;Residues: 379-619 <GE2>
A;Cross-references: GB:M14554; NID:g168823; PIDN:AAA33590.1; PID:g168824
C;Comment: This enzyme, which catalyzes the oxidation of benzendiol to
benzosemiquinone by molecular oxygen, is a multicopper oxidase containing type
1, type 2, and type 3 copper centers.
C;Genetics:
A;Introns: 86/3
C;Superfamily: laccase
C;Keywords: copper; glycoprotein; oxidoreductase
F;1-21/Domain: signal sequence #status predicted <SIG>
F;22-49/Domain: propeptide #status predicted <PRO>
F;50-619/Product: laccase #status predicted <MAT>
F;84-215/Domain: amino-terminal beta-barrel #status predicted <BB1>
F;216-372/Domain: middle beta-barrel #status predicted <BB2>
F;431-580/Domain: carboxyl-terminal beta-barrel #status predicted <BB3>
F;139,282,295,340,422,444/Binding site: carbohydrate (Asn) (covalent) #status
predicted
F;144,480/Binding site: copper (His) (type 2) #status predicted
F;146,189,191,482,548,550/Binding site: 2Cu-O cluster (His) (copper type 3)
#status predicted

Isolation and partial nucleotide sequence of the laccase gene from *Neurospora crassa*: Amino acid sequence homology of the protein to human ceruloplasmin

(multicopper oxidase/copper binding sites/genomic cloning/protein evolution)

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Communicated by Frank W. Putnam, August 6, 1986

ABSTRACT The laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) gene from *Neurospora crassa* was cloned and part of its nucleotide sequence corresponding to the carboxyl-terminal region of the protein has been determined. The gene was cloned by cDNA synthesis with a laccase-specific synthetic deoxyundecanucleotide as primer and poly(A) RNA isolated from cycloheximide-treated *N. crassa* cultures as template. Based on the nucleotide sequence of the cDNA obtained, a unique 21-mer was synthesized and used to screen a genomic DNA library from *N. crassa*. Five different positive clones were isolated and shown to share an overlapping DNA region with the same pattern of restriction sites. Sequence analysis of the common 1.36-kilobase *Sal*I fragment revealed an open reading frame of 726 nucleotides. The amino acid sequence deduced is in complete agreement with the primary structures of several tryptic peptides isolated previously from *N. crassa* laccase. The analyzed carboxyl-terminal region of laccase exhibits a striking sequence homology to the carboxyl-terminal part of the third homology unit of the multicopper oxidase ceruloplasmin and to a smaller extent, to the low molecular weight blue copper proteins plastocyanin and azurin. Based on amino acid sequence comparison between these proteins, putative copper ligands of *N. crassa* laccase are proposed. Moreover, these data further support the hypothesis that the small blue copper proteins and the multicopper oxidases have evolved from the same ancestral gene.

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belongs to the multicopper oxidases containing three distinct Cu centers known as type 1 or blue, type 2 or normal, and type 3 or coupled binuclear (1). The enzyme catalyzes the four-electron reduction of molecular oxygen to water with the concomitant oxidation of organic substrates. Laccases have been isolated from different sources such as microorganisms (2-8) and plants (9-11). Physical characterization of the proteins indicates a great variety in molecular weight, carbohydrate content, and quaternary structure. With the exception of the laccase from *Agaricus bisporus* and the tetrameric *Podospora anserina* laccase I, all native enzymes bind four copper ions per molecule, representing one type 1, one type 2, and one type 3 Cu center (12). In contrast, the other multicopper oxidases, ceruloplasmin and ascorbate oxidase, contain more than four copper ions per molecule (13-17). Hence, laccase provides the most simple system to study the active site structure and reactivity of multicopper oxidases.

Neurospora crassa laccase is excreted into the culture medium after induction with low amounts of protein synthesis inhibitors (18). The enzyme has a molecular mass of 64 kDa, with a carbohydrate content of 11-12% (4, 5). In an

attempt to determine the primary structure of *N. crassa* laccase and to study its molecular mechanisms of biosynthesis and excretion, we set out to clone the structural gene. Here we describe the strategy used to clone this gene and present part of its nucleotide sequence. The amino acid sequence deduced is compared to that of other blue copper proteins, such as ceruloplasmin, plastocyanin, and azurin.

MATERIALS AND METHODS

Organism and Conditions of Culture. *N. crassa* wild-type strain FGSC 321 (Fungal Genetics Stock Center, Kansas City, KA) was grown and induced with 2.8 μ M cycloheximide as described by Froehner and Eriksson (18).

Protein Chemical Methods. Extracellular *N. crassa* laccase was isolated essentially as described (5) from the culture medium collected 96 hr after induction. Apolaccase was prepared by dialysis of the native enzyme against 5% (vol/vol) formic acid at 4°C for at least 24 hr. The lyophilized metal-free protein was modified by S-carboxymethylation (19). Tryptic digestion of the carboxymethylated apolaccase was performed in 0.1 M ammonium bicarbonate (pH 8.5) with 2% (wt/wt) trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone; Worthington) at 37°C. After 1 hr, a second portion of trypsin was added and the mixture was further incubated for 3 hr at 37°C. Peptides were purified by HPLC on a LiChrosorb RP-18 column (0.46 \times 25 cm). The buffer system used was buffer A/0.1% (wt/vol) trifluoroacetic acid, pH 1.9, and buffer B [same as A except with 60% (vol/vol) acetonitrile]. Chromatography was carried out using a linear gradient of buffer B (0.5% per min) at 20°C with a flow rate of 1 ml/min. Absorbance was recorded at 220 nm. The peptides obtained were further characterized by amino acid analysis (Durrum Chemical, Palo Alto, CA, D-500 amino acid analyzer) and by automated Edman degradations (Beckman Sequenator model 890-B, updated).

Isolation of Poly(A) RNA. Total cellular RNA was isolated from lyophilized mycelia harvested 48 hr after induction by the method of Lucas *et al.* (20). Poly(A) RNA was selected by oligo(dT)-cellulose chromatography according to Aviv and Leder (21).

Synthesis of Oligodeoxynucleotides and Primer Extension. Oligodeoxynucleotides were synthesized according to the phosphotriester method (22, 23). Dimers were used as starting products, and synthesis was carried out on a manual solid-phase DNA synthesizer (Bachemgentec, Torrance, CA). Oligomers were fully deprotected and subsequently purified by gel filtration on Sephadex G-25 (Pharmacia), followed by gel electrophoresis (20% acrylamide/8 M urea) and chromatography on NACS 52 (Bethesda Research Laboratories).

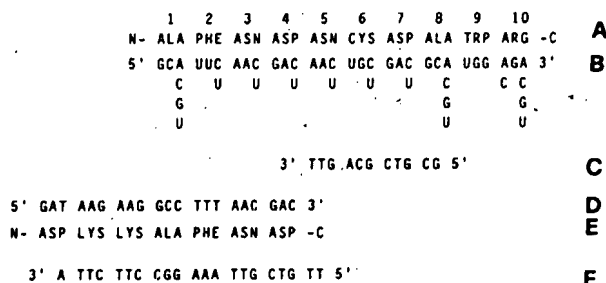
Abbreviation: kb, kilobase(s).

*To whom reprint requests should be addressed.

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DNA Sequencing. DNA sequence analysis was carried out by either the chemical cleavage (30) or the chain-termination technique (31). Single-stranded templates for the chain-termination method were obtained by molecular cloning of isolated subfragments in the single-stranded bacteriophage vectors M13mp18 or M13mp19 (32). DNA sequences were analyzed with a computer program (33).

Synthesis of Laccase-Specific cDNA by Primer Extension and Screening of a Genomic *Neurospora* DNA Library. Synthetic oligonucleotide primers provide a powerful tool for the efficient detection and transcription of specific mRNA species and thus have been often used to isolate cDNA (e.g., see refs. 34 and 35) or genomic DNA (e.g., see refs. 36 and 37) clones. To design a unique oligonucleotide suitable for priming laccase-specific cDNA synthesis, tryptic peptides were prepared from the carboxymethylated apoprotein. Purified fractions isolated by HPLC were subjected to sequence analysis (data not shown). From the tryptic decapeptide T₅ with the amino acid sequence Ala-Phe-Asn-Asp-Asn-Cys-Asp-Ala-Trp-Arg (Fig. 1A), a unique 11-mer (Fig. 1C) coding for residues 5–8 within the peptide was deduced. This primer



DNA Sequence Analysis. The 1.36-kb *Sal* I fragment common to all five different positive clones (Fig. 2) was subjected to DNA sequence analysis with the strategy outlined in Fig. 3. Both the chemical cleavage (30) and the chain-termination (31) techniques were used. The 1.36-kb *Sal* I fragment was found to contain an open reading frame of 726 nucleotides starting with the triplet GTA of one of the two *Sal* I restriction sites and ending with the termination signal TAG (Fig. 4), thus encoding the carboxyl-terminal part of a protein. The correct 21-mer (Fig. 1F) used to screen the genomic *N. crassa* DNA library could be localized readily. It is interesting to note that the designed 11-mer used for primer extension (Fig. 1C) obviously worked with one G-U mismatch. The amino acid sequence deduced from the coding region agrees completely with the primary structures of six previously isolated tryptic peptides (T_1 - T_6) of *N. crassa* laccase (Fig. 4). Hence, these results clearly indicate that the deduced amino acid sequence represents part (40–50%) of the

[†]Germann, U. A., Huber, M. & Lerch, K., 16th Meeting of the Federation of European Biochemical Societies, June 25-30, 1984, Moscow, USSR, p. 278 (abstr.).

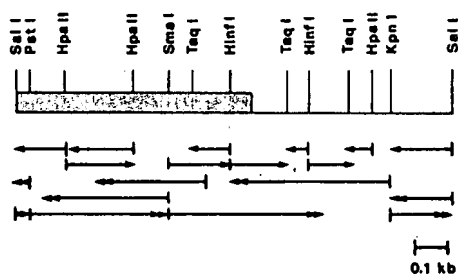


FIG. 3. Strategy of sequence analysis of the 1.36-kb *Sal* I fragment common to the five positive clones (Fig. 2). Only nonfrequent restriction sites and the sites used for sequence analysis are shown. Arrows indicate direction and extent of sequencing. For the chemical cleavage sequencing (30) (→) restriction fragments were labeled at their 5' ends and were either cut with another restriction enzyme or were subjected to strand separation. Chain-termination sequencing (31) (→) was performed using the wild-type phages M13mp18 and M13mp19 (32). Shaded bar represents the part coding for the carboxyl-terminal region of *N. crassa* laccase shown in Fig. 4.

primary structure of *N. crassa* laccase. This conclusion is further supported by the data of a preliminary sequence analysis of laccase B from the white rot fungus *Polyporus versicolor* (42). Among three fragments characterized, the active site peptide Leu-His-Cys-His-Ile-Asx-Phe indicates clear homology to the *N. crassa* laccase sequence Met-His-Cys-His-Ile-Ala-Trp. The alignment of the two peptides reveals four amino acid residues to be identical and two to be isofunctional (Leu and Met, Phe and Trp).

Sequence Comparison of *N. crassa* Laccase with Other Blue Copper Proteins. In Fig. 5, part of the amino acid sequence of *N. crassa* laccase is compared with homologous parts of human ceruloplasmin (43) and with the primary structures of plastocyanin from *Populus nigra* var. *italica* (44) and azurin from *Pseudomonas aeruginosa* (45). Ceruloplasmin is so far the only multicopper oxidase with a completely known primary structure (43). The single polypeptide chain consists of 1046 amino acid residues and exhibits a 3-fold internal homology in amino acid sequence (46, 51). Six (or seven) copper ions are bound per molecule—namely, two type 1,

one type 2, and three (or four) type 3 (13–15). Plastocyanin and azurin are low molecular weight blue copper proteins binding only one type 1 copper ion per molecule (52, 53). Amino acid sequences of azurin and plastocyanin from a wide variety of sources have been determined (52, 53). Both poplar plastocyanin and azurin from *P. aeruginosa* have been crystallized and their three-dimensional structures as well as their copper ligands are known (49, 50). Plastocyanin and azurin have been shown to be divergently related (54), and in several reports it has been suggested that the small blue copper proteins and the multicopper oxidases share a common ancestry (47, 55, 56). The amino acid sequence comparison shown in Fig. 5 further supports this hypothesis. The primary structure of *N. crassa* laccase is highly homologous to the carboxyl-terminal part of ceruloplasmin (CpC) and to a smaller extent to the other two homology units (CpN, CpM) of ceruloplasmin and to plastocyanin and azurin. In all six polypeptides compared, five amino acid residues are invariant (one Cys, two Gly, two His) and four are isofunctional having a conserved aromatic (three Phe, Trp, or Tyr) or hydrophobic (one Leu or Met) character. Among the invariant ones, three represent ligands of the type 1 copper in plastocyanin (His-37, Cys-84, and His-87) and azurin (His-46, Cys-112, and His-117). The fourth blue copper ligand in plastocyanin and azurin is Met-92 and Met-121, respectively. The corresponding residues Met-690 and Met-1031 are found only in CpM and CpC and represent the putative fourth binding sites for the two type 1 copper ions of human ceruloplasmin (51). Surprisingly, however, *N. crassa* laccase lacks a methionine residue at the homologous position. Hence, this finding implicates a type 1 copper coordination different from plastocyanin and azurin, and possibly from ceruloplasmin. In this context, it is interesting to note that stellacyanin, a small blue copper protein isolated from *Rhus vernicifera* (57) is also devoid of methionine. The nature of its fourth copper ligand is presently still a matter of debate (58).

As mentioned above, the highest overall sequence homology is found when *N. crassa* laccase is compared with the carboxyl-terminal region of human ceruloplasmin. Thus, 114 amino acid residues of the third homology unit (CpC) of ceruloplasmin can be aligned with part of the primary structure of laccase to give 27 identical and 12 isofunctional amino acid residues (Fig. 5). Most interesting, two short

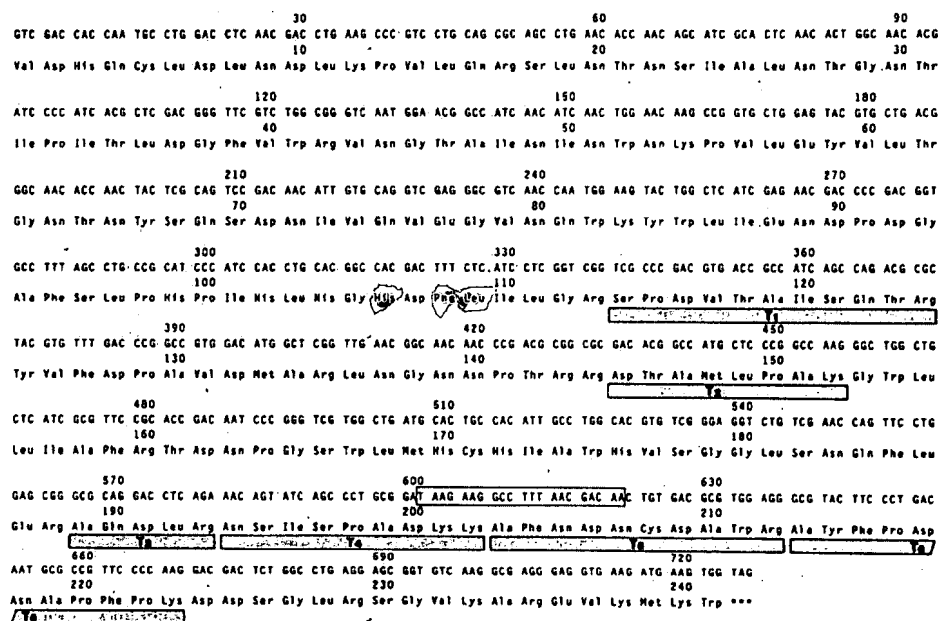


FIG. 4. Primary structure of the coding part found within the 1.36-kb *Sal* I fragment (Fig. 3) and deduced amino acid sequence. The nucleotides complementary to the 21-mer (Fig. 1F), which was used to screen the genomic *N. crassa* DNA library, are boxed. Shaded bars (T₁–T₆) indicate tryptic peptides that were isolated previously from carboxymethylated apolaccase and characterized by amino acid and sequence analysis.

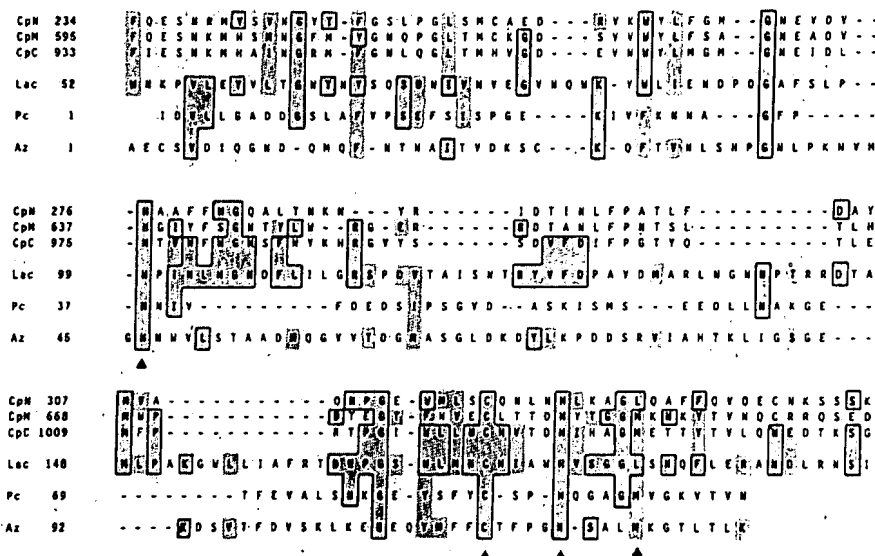


FIG. 5. Amino acid sequence comparison between part of *N. crassa* laccase (Lac) and homologous parts of other blue copper proteins. Cp, human ceruloplasmin (43); CpN, first homology unit residues 234–344; CpM, second homology unit residues 595–705; CpC, third homology unit residues 933–1046; Pc, poplar plastocyanin (44); Az, *P. aeruginosa* azurin (45). The three homology units CpN, CpM, and CpC of ceruloplasmin were aligned as described by Takahashi *et al.* (46). CpC and Pc were aligned according to Rydén (47) and Pc and Az were aligned according to Murata *et al.* (48) with some modifications maximizing the sequence homology of each primary structure to that of *N. crassa* laccase. The positions of the four copper ligands in Pc (49) and Az (50) are marked by triangles. The number on the left of each sequence identifies the position within the protein of the first amino acid residue shown. For Lac, the numbers refer to valine as 1 according to Fig. 4. Amino acid residues are abbreviated using the one-letter code. Dashes represent gaps introduced for alignments. Boxes relate amino acid residues of Lac with identical residues in CpN, CpM, CpC, Pc, and/or Az. Shaded areas, isofunctional residues (D and E; K and R; T and S; F, W, and Y; I, L, M, and V).

stretches show extensive sequence homology. A computer analysis using the unitary matrix indicated maximal homology scores of 6 and 9 for the segments presented in Fig. 6 A and B, respectively (N. Takahashi and F. W. Putnam, personal communication). The remarkable similarity of these two regions suggests that they play an important role in the formation of the active site of multicopper oxidases. In particular, they could provide ligands to all three different types of copper in both enzymes. This suggestion is consistent with the observation of Raju (59) that 50% of the non-blue copper of the ceruloplasmin molecule resides in the carboxyl-terminal tryptic peptide of 11 kDa. As pointed out above, at least three invariant type 1 copper binding residues are present within these two stretches. The fourth type 1 copper ligand in *N. crassa* laccase is currently not known (see above). It is conceivable, however, that the nearby Met-169 of *N. crassa* laccase might be involved in copper binding. The

idea that also type 2 or type 3 copper ligands could be provided by the two homologous regions shown in Fig. 6 is based on earlier reports. The sequence containing the histidine cluster of ceruloplasmin (Fig. 6A) has been shown to be homologous to that providing ligands to the non-blue copper site in Cu/Zn-superoxide dismutase (51). Furthermore, using the coordinates of the known three-dimensional structure of poplar plastocyanin (49), Rydén recently presented a three-dimensional model of the copper active site of human ceruloplasmin (47). The model involves the carboxyl-terminal region (amino acid residues 932–1040; ref. 43) and proposes His-975, Cys-1021, His-1026, and Met-1031 as type 1; His-1022 and His-1028 as type 2; and His-978, His-980, His-982, and His-1020 as type 3 copper ligands. The amino acid sequence comparison of ceruloplasmin with laccase supports this model with respect to the type 1 and the type 3 copper ligands. However, no histidine residue is found in

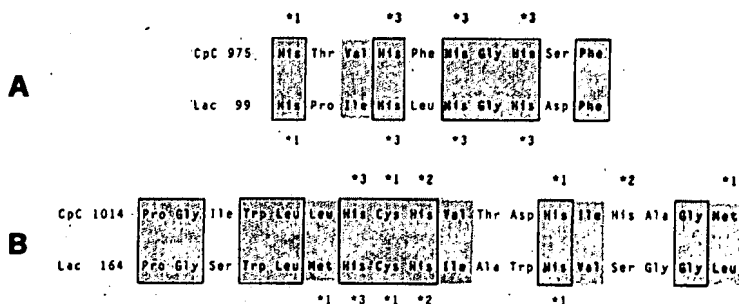


FIG. 6. Amino acid sequence comparison of two highly homologous regions (A and B) of *N. crassa* laccase (Lac) and the carboxyl-terminal homology unit (CpC) of human ceruloplasmin (46). Numbers on the left of each sequence identify the positions within the proteins of the first residues shown. For Lac, numbers refer to valine as 1, according to Fig. 4. Identical amino acid residues are boxed and isofunctional amino acid residues are shaded (see Fig. 5). Potential ligands to the three different types of copper are indicated by *1, *2, and *3, respectively. The assignment of the copper ligands for *N. crassa* laccase is based on that proposed earlier for CpC (47).

laccase that is homologous to the suggested type 2 copper binding His-1028 of ceruloplasmin. Hence, our data implicate that either the structures of the type 2 site in ceruloplasmin and laccase are different or the homologous type 2 ligands are closer to the amino terminus than suggested. It is clear that the complete amino acid sequence of *N. crassa* laccase and those of other multicopper oxidases are required to fully define the amino acid residues involved in the binding of the active site copper.

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